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Cultivation of variola virus in the suckling mouse.

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Infantile animals are particularly suited to the cultivation of many viruses. The suckling mouse has acquired the greatest significance in this respect. It is being utilized in the study of numerous viruses belonging to the arthropod-borne encephalitides (1, 2, 14 to 19), in investigations of the viruses of foot-and-mouth disease (11), yellow fever (3), Theiler (13) and Coxsackie (12). Recently, the viruses of hog cholera (9), rabbit myxoma (24) and rabbit fibroma (10) were induced to propagate in suckling mice. In addition, Newcastle disease virus (22), the viruses of herpes simplex (25), hepato-encephalomyelitis (26), Nairobi sheep disease (21), stomatitis vesicularis (23), as well as REO viruses (28) recently described by Sabin, may be grown in infant mice. Certain poliovirus strains also reproduce in suckling mice after adaptation (20). The same applies to the virus of distemper (27).

Of the pox pathogens, this experimental animal lends itself well to the propagation of the viruses of ectromelia, vaccinia (7, 29) and those of original cow pox (8).

The present study was designed to test the suitability of the infant mouse for cultivation of variola virus.

Under natural conditions, variola virus is limited to man and the monkeys. It may not be maintained by passages through the usual laboratory animals without loss of specificity. On the other hand, cultivation in incubated chicken eggs has been successful (4-6).

A study by Sarkar, Neogy and Lahiri (1959) is the only instance of variola virus propagation in suckling mice known to us. The authors investigated the behavior of vaccinia and variola virus in adult and infant mice. Both viruses reproduced in suckling mice, with vaccinia virus revealing a greater virulence for the animals (29).

Material and methods.

Our starting material consisted of dried pustule contents and scabs. This material came from variola patients admitted to the City Fever Hospital in Bombay during the Indian variola epidemic in the spring of 1958.

For the initial passage, the pustules and scabs were ground, mixed 1:5 with m/90 phosphate buffer, pH 7.3, followed by addition of penicillin and streptomycin, and separated in a low-revolution centrifuge. We injected 0.1 ml intraperitoneally per animal. The same virus material induced generalized variola in monkeys.

Both during initial cultivation and in the passages, we used the same inoculum on several litters of different families. The animals were products of breeding on the premises, free of latent viral infections. The animals' age fluctuated between 1 and 3 days, depending on the test.

Demonstration of virus from the organs of expired, diseases or in-apparently infected suckling mice was carried out on the chorioallantoic membrane (CAM) of 10-day incubated chicken eggs. Virus titrations proceeded in the same manner. Dilutions were related to the tissual raw material. The titer was computed for 0.1 ml according to Reed and Muench's determination of ID_{50}. The technique of inoculation, evaluation and titration has been published previously (7, 30, 31).

The incubated chicken egg was also employed in the testing of stability of variola virus during suckling mouse passages. Criteria of evaluation were 1. incubation time, 2. the macroscopic and microscopic appearance of chorioallantoic primary and secondary foci, 3. percentage, quantity and time of generalization, 4. death rates of infected chick embryos (comparison between egg ID_{50} and egg LD_{50}).

Tests and results.

In the initial passage, the ground variola pustule material was injected into 5 litters of 3-day-old animals from different families. The titer of the inoculum was $10^{-2.5}$ egg ID_{50}. Further passages through identical numbers of mice were made with a 20% combined extract of lungs, liver, spleen and kidneys, either from expired animals or those sacrificed between the 8th and 10th day p.i., if no fatalities had occurred. Twenty passages through infant mice were carried out in this manner. Table 1 reflects the behavior of variola virus during these passages.

All animals infected for the initial passage died between the 3rd and 8th day p.i. The appearance at section resembled hemorrhagic septicemia with minute petechial extravasations in the kidney and spleen, while animals succumbing between the 7th and 8th day also revealed these

hemorrhagic foci in the lungs. All animals had ascites with major accumulations of fluid in the abdominal cavity. In animals expiring on the 6th day, virus was found predominantly in the lungs ($10^{-4.5}$) and the kidneys ($10^{-2.5}$ to $10^{-5.8}$). Liver titers fluctuated between $10^{-0.5}$ and $10^{-2.5}$, depending on the animal, and between $10^{-0.5}$ and $10^{-1.0}$ in the spleen. The clinical process remained unchanged up to the 3rd passage. The animals died between the 5th and 12th day p.i. Pathological anatomy was again characterized by a large accumulation of fluid in the abdominal cavity. The hemorrhagic symptoms abated, however, and were limited to isolated renal and pulmonary foci. The virus titers in the organs decreased up to the 3rd passage. Wide fluctuations in the organs' virus content were noted within the various litters. Radical changes were brought about by the 4th passage. The animals no longer died of infection. While a few litters showed occasional light impairment in general health between the 6th and 9th day p.i., they made rapid recoveries. Virus was demonstrated in all passages. The lungs were always affected most, reaching infectivity titers up to $10^{-4.5}$ egg ID_{50} in some animals on the 7th day p.i. The fact that the lungs are a preferred organ for the propagation of variola virus in the infant mouse was shown clearly by findings after 32 days. At this time, the only demonstrable viral concentrations were found in the lungs. A second small change was note between the 11th and 18th passage: Some litters died between the 5th and 19th day p.i., others survived as before without symptoms of disease. Organs of expired animals contained much more virus than those of healthy mice. The symptomatology did not vary between the animals of one litter. Increasing numbers of passages were accompanied by a general rise in fatal cases. Most litters succumbed after the 19th and 20th passage. These animals yielded virus titers up to $10^{-5.5}$ from their lungs.

In another experiment we tested the suitability of one-day-old mice. We inocu'ated virus material of the 11th to 20th suckling mouse passage. One-day-old mice invariably proved more susceptible to variola virus than 3-day animals. But even among these there were some litters that remained healthy, although virus could be isolated from their organs, especially the lungs, up to the 45th day p.i.

We pursued this problem further. A major experiment utilized 292 1-day mice from 31 different litters of our institutional Breed II, which were infected with a 20% pulmonary extract of the 18th infant mouse passage (egg ID_{50} $10^{-4.0}$). Litters were selected according to the principle of "accidental distribution." Twenty-three litters died between the 5th and 8th day specifically of variolar infection, 8 litters survived without signs of disease. During an observation phase of 36 days their lungs yielded virus with titers between $10^{-0.5}$ and $10^{-4.5}$.

Viral stability during sustained mouse passages was tested by reinoculation on chick embryo chorioallantoic membranes. The typical behavior of variola virus in chick embryos did not change between the 1st and 20th infant mouse passage, with the exception of a slight rise in virulence (greater percentage of generalization and death rates, greater quantity of generalization). Specific deviations from vaccinia infections of the chick embryo, shown with particular clarity in the appearance of the chorioallantois, were always retained. Figs. 1 to 3 offer comparisons of different variolar membranes after a contrasting number of passages with a typical chorioallantois of vaccinia.

Discussion of results.

The suckling mouse represents an inexpensive, small experimental animal for the cultivation of variola virus. Propagation of virus succeeded in 20 successive passages. The virus remained stable with respect to its biological properties in the chick embryo.

The infection was invariably lethal in the first few mouse passages. Viral virulence abated with increasing passages, resulting in wide fluctuations in resistance and susceptibility among different mouse families. Some families seemed to possess a congenital non-specific resistance to variola infection, warding off a fatal termination. In these animals the virus reproduced without evoking clinical manifestations. After 18 infant mouse passages, the virus experienced a general restoration of virulence, causing most litters to succumb between the 5th and 8th day p.i.

One-day-old suckling mice proved more susceptible to variolar infection than 3-day animals.

During the first few passages the appearance of specifically expired mice at section resembled hemorrhagic septicemia with bleeding in the kidneys, spleen and lungs. Later passages were characterized by ascites with large accumulations of fluid in the abdominal cavity and hydrothorax. The pathologic-anatomical picture of variola in suckling mice thus recalls a vaccinia infection (7). However, the hemorrhagic conditions was not as distinctly pronounced and tended to disappear in subsequent passages. Still, ascites and hydrothorax dominated in the manner of vaccinal infections. Dissimilarities ought to be attributable to greater virulence of vaccinia virus for infant mice.

The lungs were a favored organ for variola virus propagation. Animals that succumbed specifically to the infection yielded viral titers up to $10^{-5.5}$ egg $ID_{50}/0.1$ ml. These values were far in excess of those of other organs, e.g. the liver, spleen and kidneys. Infected animals without clinical symptoms also harbored relatively high concentrations of virus in the lungs. These findings are important for the pathogenesis of variolar infections. The suckling mouse offers a medium for further study of the genesis and dissemination of variola.

Another important and interesting observation involves the protracted persistence of variola virus in infected, but clinicall. healthy animals. The lungs of such animals regularly yielded infective virus during a period of 45 days p.i. The animals were not examined beyond this point. It may be that the demonstration of virus in the lungs is possible for even longer periods. The findings of Sarkar, Neogy and Lahiri (29) are significant in this connection. The authors infected suckling mice intracerabrally with variola virus. Animals surviving the infection clinically unimpaired yielded variola virus from the brain 62 days p.i. Our results point in the same direction. The prolonged persistence of infective variola virus in clinically healthy animals raises new epidemiological problems demanding further attention. Data recently published by Alivisatos et al. (32), relating to the demonstration of human variola 38 days after inception, are worthy of mention in this connection.

Summary.

Variola virus from human pustules and scabs was induced to grow intraperitoneally in suckling mice and was continued in sustained passages. It remained stable during 20 passages with respect to its biological properties in the chick embryo. One-day-old mice were more susceptible than 3-day animals.

Variable resistance to variolar infection was seen among different mouse families. The virus propagated in more resistant animals without causing clinical manifestations.

The lungs were a preferred organ for variola virus propagation.

Infective variola virus was isolated from the lungs of infected, but clinically healthy mice for 45 days.

Illustrations.

Fig. 1. Appearance of chorioallantois infected with vaccinia virus on the 4th day p.i.: Wide areal primary foci with distinct central necrosis, 4 / generalization, secondary foci also with central necrosis.

Fig. 2. Appearance of chorioallantois infected with variola virus on the 6th day p.i.: (from the first suckling mouse passage, lungs): Proliferative primary foci without central necrosis, concentric zones of turbidness around the nodes of proliferation, beginning generalization.

Fig. 3. Appearance of chorioallantois infected with variola virus on the 7th day p.i.: (from the 18th suckling mouse passage, lungs): Lower right, confluent primary foci without central necrosis, 3 / generalization along the vessels; secondary foci, also without central necrosis, are proliferative nodes with peripheral zones of turbidness.

Table 1. Behavior of variola virus in 3-day-old mice during 20 intraperitoneal passages.

Passages	Results of infection	Post mortem appearance Time p.i.	Post mortem appearance Findings	Viral content of organs (neg. log$_{10}$ egg ID$_{50}$/0.1 ml) Time of exam.	lungs	Kidneys	Liver	spleen
Initial	Death between 3rd & 8th day	8th day	Ascites, hemorrh. in kidneys, spleen, lungs	6th day	4.5	2.5-5.8	0.5-2.5	0.5-1.0
2d-3rd	Death between 5th & 12th day	8th day	Ascites, only isolated hemorrh. in kidneys & lungs	6th day	1.5-5.2	2.0-4.5	1.5-3.7	0.5-1.5
4th-10th	All litters survive	7th day / 32d day	negative / negative	7th day / 32d day	1.5-4.5 / 0.6	ng-0.5 / neg	ng-1.0 / neg	ng-0.5 / neg
11th-18th	Some litters die between 5th & 19th day	5th to 19th day	Ascites, hemorrh. foci in lungs	7th day (exitus)	5.0	3.0	2.5	2.5
	Some litters survive	21st day	negative	10th to 21st day	0.5-4.0	ng-1.0	ng-0.5	..5-0
19th & 20th	Majority of litters dies between 5th & 8th day	8th day	Ascites, hydrothorax	8th day	4.0-5.5	2.0-2.5	1.0-2.0	1.5-2.0
	Some litters survive	21st day	negative	21st day	0.5-4.5	ng-1.0	neg	neg

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